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PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP2004/008097	20.07.2004	22.07.2003

International Patent Classification (IPC) or both national classification and IPC
C07D475/14

Applicant
DSM IP ASSETS B.V.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA"). However, this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of three months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/EP2004/008097

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This opinion has been established on the basis of a translation from the original language into the following language , which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ in written format

   ☐ in computer readable form

   c. time of filing/furnishing:

   ☐ contained in the international application as filed.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. II Priority

1. ☒ The following document has not been furnished:

   ☒ copy of the earlier application whose priority has been claimed (Rule 43*bis*.1 and 66.7(a)).

   ☐ translation of the earlier application whose priority has been claimed (Rule 43*bis*.1 and 66.7(b)).

   Consequently it has not been possible to consider the validity of the priority claim. This opinion has nevertheless been established on the assumption that the relevant date is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. ☐ It has not been possible to consider the validity of the priority claim because a copy of the priority document was not available to the ISA at the time that the search was conducted (Rule 17.1). This opinion has nevertheless been established on the assumption that the relevant date is the claimed priority date.

4. Additional observations, if necessary:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2,4,6-12
	No:	Claims	1,3,5-7
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-12
Industrial applicability (IA)	Yes:	Claims	1-12
	No:	Claims	

2. Citations and explanations

see separate sheet

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY (SEPARATE SHEET)

International application No.
PCT/EP2004/008097

Reference is made to the following documents:

D0: US 2 324800 (cited in the application)
D1: US 2 797 215 (cited in the application)
D2: US 2 603 633 (cited in the application)
D3: EP 464 582 (cited in the application)
D4: EP 730 034
D5: US 6 150 364
D6: EP 1 048 668

Re Item V

1) Claim 1 is not clear (Article 6 PCT)

1.1) Claim 1 does not specify all of the essential features needed to define the invention (Guidelines 5.33).

The technical problem underlying the present application is seen in the provision of a superior process for the purification of riboflavin (cf. page 2). The invention is based on the finding that the DNA content of riboflavin crystals can be significantly reduced (cf. page 3).

Therefore, the claimed process only solves the technical problem when applied to riboflavin comprising substantial amounts of DNA. However, the present claim 1 does not specify the source of riboflavin and comprises the treatment of synthetic riboflavin (see also D0, teaching that 'obviously, the purification of crude synthetic riboflavin is a very different problem from that of isolating the natural product. Although the impurities in the synthetic product constitute only a minor proportion of the whole mass, they are of a very different nature from the substances which in natural riboflavin occurs' [col 1, line 54 - col 2 line 5]).

1.2) The present process according to claim 1 relates to a process in which the first crystalline form of riboflavin is thermodynamically less stable than the second crystalline form. Thereby, the claim defines the invention by the result to be achieved. In view of the fact that the said result is achieved (only) by inducing the precipitation of the first form of riboflavin by seeding the fermenter with crystals of appropriate crystal form, the area defined by the claims is not as precise as the invention allows.

2) The subject-matter of present claims 1, 3 and 5-7 is not new (Article 33(2) PCT).

2.1) D1 relates to a process for the preparation of form A riboflavin (anhydrate I; cf. page 7 of the present description).

The process of D1 encompasses the following steps: precipitated crude riboflavin of different origin (e.g. crystalline riboflavin of types other than pure Type A) is dissolved in an aqueous alkaline solution. The solution is then filtered and the filtrate is acidified with an acid, whereupon riboflavin immediately separates in a form which may be minute crystals of undetermined type i.e. Type B or lathlike crystals of Type C. This slurry which cannot be effectively filtered is then boiled to convert the crystals therein to Type A, after which it can be filtered and dried (cf. column 2, lines 36-60).

The acid is added to the aqueous alkaline solution containing riboflavin in quantities sufficient to reduce the pH preferably within the range of about 3.5-5.5. Any acid can be used for this step, particularly good results being obtained with sulfuric, nitric, hydrochloric and-phosphoric acids. The slurry obtained is then boiled to convert the precipitated riboflavin into the desired long fibrous needles of Type A.
It has been found that due to variations in riboflavin precursor filter cakes, the resulting Type A riboflavin crystals are oftentimes very difficult to filter and conversions and yields vary widely. To eliminate these difficulties, a settling and decanting step can be inserted in the process just following acidification of the alkaline extract. The precipitate settles rapidly to a volume of about one-fourth of the whole. The supernatant liquor can then be decanted and water equal to about one-half the volume of the decanted added before the slurry is boiled to convert the riboflavin to Type A crystals. By the insertion of these settling and decanting steps, the average purity of the Type A crystals can be increased and the filtration rate of the Type A crystals greatly accelerated (cf. column 3, lines 50-75).
After conversion to Type A crystals is complete, the slurry is cooled to a temperature below about 25 C. The crystals are then filtered and washed with water until free of salt. The pure Type A riboflavin crystals are then dried (cf. column 4, lines 15-29). This generic teaching destroys the novelty of the present claims.

In example II a riboflavin precursor filter cake, was slurried in sodium hydroxide until the riboflavin precursor was dissolved. The slurry was then filtered and the filtrate was then acidified with sulfuric acid to a pH of 4.5 precipitating the riboflavin as **small ball-like globules**. The solid-phase was allowed to settle and the supernatant was decanted and replaced by water. The slurry was then boiled and seeded at the boiling point, with Type A crystals. The boiling was continued for two hours at which time the riboflavin had been converted to the **long fibrous needles** characteristic of Type A. The slurry was then cooled and washed with water until free of sulfate. The yield of Type A crystals was 80% and the crystals had a purity of 95%. This example destroys the novelty of the present claims 1, 5-7.

Furthermore, in example III, riboflavin crystals prepared by the method of U. S. Patent 2,603,633, (D2; Type C crystals; tetrahydrate, cf. present page 7) were converted to Type A crystals by the following procedure: Sufficient Type C crystals to give 20,000 grams of riboflavin by assay were dissolved in 270 gallons of 0.1 N sodium hydroxide solution at room temperature and the solution was pumped into a crystallizer equipped with a steam coil for heating. The pH of the slurry was then adjusted to 3.5-4.0 with dilute sulfuric acid. It was then seeded with Type A crystals and heated to 95-98 C. with steam. Heating was continued until the crystalline material in the slurry was completely converted into long silken needles when viewed under the microscope. As soon as conversion was complete, water was put through the heating coil and the slurry cooled overnight until the temperature fell to below 25 C. The crystals were then filtered and washed with condensate water until free of sulfates. The filter cake was then broken up, dried in a warm air drier and finally micropulverized.
According to the above mentioned process of D2, the type C crystals are obtained by crystallisation (cf. claim 2 of D2). Consequently, this example destroys the novelty of the present claims 1, 3 and 5-7.

2.2) D3 relates to a method of purifying riboflavin which has been prepared by microbial fermentation, by means of which method such ferment-produced riboflavin can be obtained in a grade making it suitable for addition to foodstuffs and to pharmaceutical preparations. Furthermore, D3 provides a simple method of substantially raising the riboflavin content of ferment-produced riboflavin.
The said process relates to a method of purifying ferment-produced riboflavin, wherein the impure riboflavin is suspended in water or dilute aqueous acid, and

the suspension is heated at a temperature of from 75 DEG to 130 DEG C. and preferably from 80 DEG to 120 DEG C. for from 0.3 to 3 hours and preferably from 1 to 2.5 hours, with stirring, after which the reaction mixture is cooled and the crystals thus formed are isolated by known methods.
According to D3, such heating in water or dilute aqueous acid causes a change in crystal structure, as is evident from the fact that the mass of crystals becomes thick and pasty. Stirring is thus essential. Further heating causes a normal suspension to reform from which the riboflavin, now purified by crystal transformation can be isolated in conventional manner.
The heating method proposed in D3 makes it possible to improve the purity of riboflavin from about 96% to about 99%, from about 90% to about 97% and from about 65% to about 80%, by weight. The purifying effect can be intensified by adding to the water from about 0.05 to 10% and preferably from 0.5 to 3% w/w of a water-soluble acid, preferably a mineral acid such as H2SO4, H3PO4 or HCl.
By appropriately heating the impure vitamin B2 in dilute aqueous acid it is possible, for example, to increase the riboflavin content from about 96% to 100%, from about 90% to about 97% and from about 65% to about 90%, by weight.
By dilute aqueous acid a solution of a water-soluble acid, preferably a mineral acid which has no adverse effect on the riboflavin molecule is meant. Examples of suitable water-soluble acids are organic acids such as formic acid, tartaric acid, citric acid and acetic acid, and mineral acids such as sulfuric acid. hydrochloric acid and phosphoric acid (cf. column 2, lines 6-56).
The first precipitation step (a) according to the present claims is implicitly disclosed by the reference in D3 to prior art process for the preparation of the crude starting material (riboflavin can be obtained simply by concentrating the culture fluid, by decanting the culture fluid once or a number of times in a special manner or by producing riboflavin in a special culture and heating an aqueous solution thereof, from which the solids are separated and from which the riboflavin is then isolated by crystallization (cf. column 1 of D3). Furthermore, it is clear from the present description that in the course of the progressing fermentation the crystallisation of riboflavin in the fermenter cannot be avoided (cf. present page 7).

D3 does also not mention the thermodynamical stability of the starting or end product. However, it is clear that the first form is less stable than the later form because, otherwise, no transformation would be possible into any second crystalline form being thermodynamically more stable (see also present application page 8, lines 14). In view of the similarities of the process of D3 with

the one of D1, which produces form A, it must be assumed that in D3 also type A crystals (anhydrate I) are produced.
Consequently, the above teaching of D3 destroys the novelty of the present claims 1 and 5-7.

3) In view of lacking novelty of the single independent claim 1, inventive activity can not be assessed definitively. However, it appears that also the combination of claim 1 with any of the formally novel dependent claims does not yield inventive subject-matter (Article 33(3) PCT). The reasons being the following:

3.1) D3 appears to represent the closest prior art (cf. above).

The claims 8-10 appended to claim 1 differ from the process of D3 in that the crystal type of the first crystalline form of riboflavin in the fermenter is controlled by the addition of seed crystals of appropriate form.

The technical problem underlying the present application is seen in the provision of an improved process for the purification of DNA contaminated riboflavin.

The problem is solved by controlling the first crystalline form of riboflavin (a) in the fermenter.

In view of the fact that in D3 the acid treatment causes a change in crystal structure, it must be assumed that the control of crystal structure of the first crystalline form is only necessary when the first form crystallizing in the fermenter is form A (anhydrate I), known as the stable form of riboflavin (cf. D5, column 1, lines 28-33 and column 5, lines 49-61; or D6, page 3, [14]-[17]).
In view of the excellent results achieved by D3 (improvement in purity from 96 to 100%), the skilled person would apply the process of D3 on any ferment-produced riboflavin. However, should the skilled person realize, the acid treatment of a certain ferment-produced riboflavin does not cause a change in crystal structure, he would modify the process of D3 in order to achieve the said change in crystal structure as disclosed in D3. Therefore, the skilled person would try to control the crystal form of riboflavin precipitating in the fermenter.
It appears to belong to the common general knowledged of the relevant skilled in the art that seeding with crystals of a desired crystalline form induces crystallization in the said form. As support for this, document D1 can be cited

where in the final process step seed crystals of form A are used to accelerate the crystallization of the desired form A (cf. D1, column 4, lines 9-11 and example III). Consequently, the skilled in the art would use seed crystals in the present step (a) of a crystal form being different from the stable form A (anhydrate I).
Thereby, the skilled person arrives at the present process according to claims 8-10.

3.2) For the further assessment of inventive step also document D5 appears to be relevant. D5 relates to a process for the recovery and purification of riboflavin which has been produced by a fermentation method, i.e. is present in the so-called fermentation broth after the fermentation has been effected to a satisfactory extent, preferably to completion (said broth being hereinafter referred to as the "post-fermentation broth"), which process affords a riboflavin product of at least an acceptable quality appropriate to its intended subsequent use.
The said process for the recovery and purification of riboflavin from a post-fermentation broth is characterized by the essential steps of
(I) heating said post-fermentation broth at a temperature of about 45 DEG C to about 120 DEG C over a duration of about 10 minutes to about 2 hours for pasteurization,
(ii) centrifuging the so-pasteurized broth one or more times to obtain a product consisting largely of riboflavin,
(iii) treating the product of the previous step with aqueous mineral acid at a temperature of about 80 DEG C to about 130 DEG C over a duration of about 30 minutes to about 24 hours and
(iv) collecting the riboflavin from the aqueous acid medium of the previous step by filtration and washing it with water, and the optional step of
(v) drying the collected washed product of the previous step, to afford riboflavin of substantial purity (cf. page 2 of D4).

The purpose of the above-described process step (iii) is to hydrolyse the biomass present as an undesired impurity and to thereby convert it into a soluble form in the aqueous mineral acid. At the same time any DNA present is also degraded (hydrolysed), and high molecular weight impurities, such as proteins and polysaccharides, are decomposed and also converted into soluble forms. The riboflavin remains in substantially undissolved form on completion of this process step (cf. page 3, lines 52-56 of D4).